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Characterization of histamine H_1-receptor binding peptides in guinea pig brain using [^{125}I]iodoazidophenpyramine, an irreversible specific photoaffinity probe

(photoaffinity labels/mepyramine derivative)

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ABSTRACT **Aminophenpyramine—i.e., *N*-{5-[2-(4-aminophenyl)ethanamidopentyl]}-*N'*-(4-methoxybenzyl)-*N*-methyl-*N'*-(2-pyridinyl)-1,2-ethanediamine, a derivative of mepyramine (pyrilamine), a typical antagonist of histamine at its H_1 receptor—was synthesized and converted into [^{125}I]iodoazidophenpyramine, a potential photoaffinity probe for the H_1 receptor. In the dark, reversible binding of this probe to cerebellar membranes occurred with a K_d of 1.2×10^{-11} M and a B_{max} of 240 fmol/mg of protein and was inhibited by various H_1-receptor antagonists with the expected potencies. These features establish the compound as one of the most potent H_1-receptor antagonists known so far. Upon UV irradiation, 5% of the bound radioactivity was covalently incorporated into cerebellar membrane polypeptides as shown by standard NaDodSO$_4$/PAGE. Two bands of 47 and 56 kDa were consistently labeled, labeling being prevented by various H_1-receptor antagonists with the expected potencies and stereoselectivity. In the presence of protease inhibitors, labeling of the 56-kDa peptide increased at the expense of the 47-kDa peptide, suggesting that the latter was produced by hydrolysis of the former under the action of membrane proteases. In the absence of 2-mercaptoethanol, a band of 350–400 kDa appeared, apparently at the expense of the lighter bands, suggesting that the latter might be linked by one or more disulfide bridges to a higher molecular mass complex. We propose that at least part of the ligand binding domain of the histamine H_1 receptor resides within a subunit of apparent molecular mass 56,000.**

Histamine is a messenger molecule mainly released by neurons and mast cells that affects a large variety of target cells by interacting with three pharmacologically distinct subclasses of receptors termed H_1, H_2, and H_3 (1). The H_1 receptor has been labeled in crude membranes with the reversible ligand [^{3}H]mepyramine (2, 3). More recently, ^{125}I-iodobolpyramine, a highly specific probe chemically derived from mepyramine, has been developed for a more sensitive assay (4, 5). These reversible probes have enabled investigations into some physicochemical properties of the membrane-bound H_1 receptor, such as modulation of ligand affinity by cations, guanyl nucleotides (6), or thiol reagents (7, 8). Its apparent molecular mass in brain (9) and liver (10) has been determined by target size analysis. In addition, the receptor has been solubilized recently (11) and its glycoprotein nature demonstrated (12).

Photoaffinity probes constitute valuable tools to study the receptor structure. Photolysis of nonradioactive azide derivatives of histamine (13) or ketanserin (14, 15) were shown to block irreversibly H_1 receptors. To date, however, no results have appeared regarding the use of these probes in radioactive form.

We now describe the chemical synthesis and biological characterization of [^{125}I]iodoazidophenpyramine (^{125}I-APP), a highly potent, histamine H_1-receptor antagonist derived from mepyramine. Upon irradiation, ^{125}I-APP was covalently incorporated into the H_1 receptors of guinea pig brain membranes, leading to the characterization of the ligand-binding peptides of this receptor.

MATERIALS AND METHODS

Materials. Na^{125}I (usually 2000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham, and $NaNO_2$ and gelatin were from Prolabo (Paris). The drugs and their sources were: cimetidine, SK&F 94461, and burimamide from Smith Kline & French (Welwyn, U.K.); triprolidine and (+)- and (−)-chlorpheniramine from Burroughs Wellcome; mepyramine from Specia (Paris), mianserin from Organon; and ranitidine from Glaxo (Paris). Electrophoresis standards were from Bio-Rad, and reagents were from Eastman Kodak. 2-Mercaptoethanol, NaDodSO$_4$, protease inhibitors, NaN_3, and chloramine-T were from Sigma.

Synthesis of Aminophenpyramine. The amine SK&F 94461, or *N*-(5-aminopentyl)-*N'*-(4-methoxybenzyl)-*N*-methyl-*N'*-(2-pyridinyl)-1,2-ethanediamine (5) (1.8 mmol), was dissolved in dry CH_2Cl_2 (20 ml), added to a stirred solution of *p*-aminophenylacetic acid (5.6 mmol) in dry CH_2Cl_2 (60 ml), and cooled to 0°C; *N,N'*-dicyclohexylcarbodiimide (2.8 mmol) was added, and the mixture was stirred for 30 min at 0°C and then at 21°C for 3 hr. *N,N'*-Dicyclohexylurea was removed by filtration, and the filtrate was evaporated under reduced pressure to furnish the product—aminophenpyramine, *N*-{5-[2-(4-aminophenyl)ethanamidopentyl]}-*N'*-(4-methoxybenzyl)-*N*-methyl-*N'*-(2-pyridinyl)-1,2-ethanediamine—as an oil (0.845 g). The latter was treated with oxalic acid in ethanol and diluted with diethyl ether to furnish aminophenpyramine monooxalate as a granular solid (0.25 g, 54%), mp 78–83°C. Its identity was confirmed by ^{1}H NMR spectroscopy.

Synthesis of ^{125}I-APP. The scheme used for synthesis is shown in Fig. 1. Aminophenpyramine (10 μg/10 μl of water; 17 nmol) was mixed in a polypropylene tube with 40 μl of 1 M sodium acetate buffer (pH 5.6). Na^{125}I (10 μl; 1 mCi) and then chloramine T (44 nmol/10 μl) were added. After 1 min the reaction was stopped with $Na_2S_2O_5$ (88 nmol/17 μl), and the mixture was analyzed by HPLC (C_{18} μBondapak; Waters Associates). The mobile phase was MeCN/10 mM NH_4AcO, pH 4.2, 1:1 (vol/vol), and the flow rate was 1 ml/min.

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Abbreviation: ^{125}I-APP, [^{125}I]iodoazidophenpyramine.

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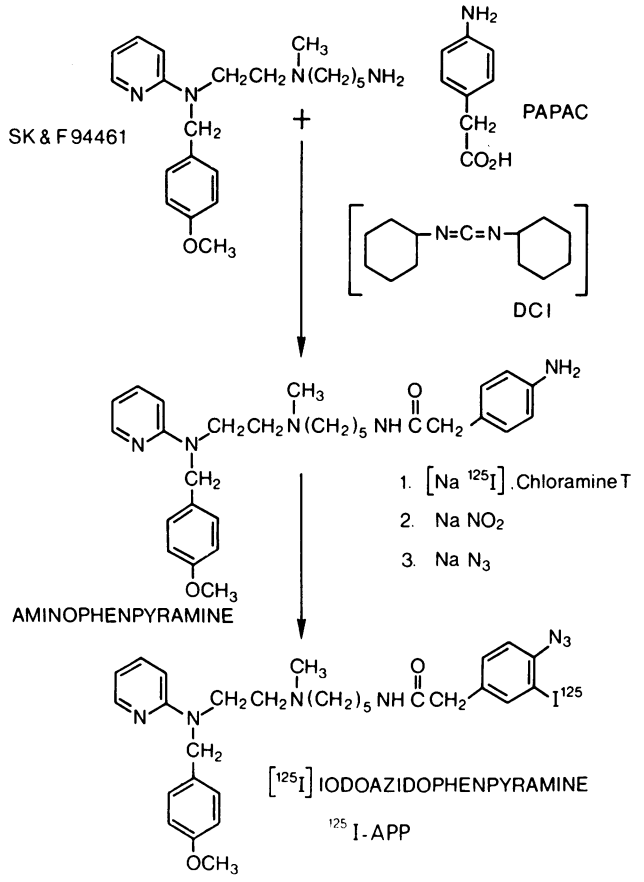

FIG. 1. Scheme for the synthesis of ^{125}I-APP, a photoaffinity probe. PAPAC, *p*-aminophenylacetic acid; DCI, *N,N'*-dicyclohexylcarbodiimide.

^{125}I-substituted aminophenpyramine ([^{125}I]iodoaminophenpyramine) was the major peak eluted (usually containing >50% of the initial radioactivity), of which a 300-μl sample was mixed with 17 M AcOH (30 μl) and $NaNO_2$ (22 μmol/30 μl) for 2 min at 0°C. NaN_3 (27 μmol/35 μl) was added for 3 min at 20–22°C, and the reaction mixture immediately was submitted to HPLC as described above. [^{125}I]Iodoaminophenpyramine (retention time, 10 min) was found to be completely transformed; the major product, ^{125}I-APP (retention time, 21 min), was collected and stored at 4°C in the dark for at least 1 month without losing its binding properties. Extreme care was taken to work under dim light during the azidation and HPLC steps.

Membrane Preparation. Cerebella from male Hartley guinea pigs (200–300 g) were homogenized with a Polytron blender in 40 vol of cold 50 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.5). After centrifugation at 260 × *g* for 1 min, the resulting supernatant was recentrifuged at 20,000 × *g* for 30 min, and the final pellet was rinsed with 2 ml of cold buffer and stored at −80°C. In the experiments with protease inhibitors, the buffer was supplemented with bacitracin (0.1 mg/ml), leupeptin (10 μg/ml), pepstatin A (0.1 μg/ml), soybean trypsin inhibitor (10 μg/ml), phenylmethylsulfonyl fluoride (0.1 mM), and EDTA (10 mM). For binding assays, the pellets were resuspended in buffer, and the protein concentration was determined by the method of Lowry with bovine serum albumin as standard.

^{125}I-APP Binding. Assays were performed in polypropylene tubes, and gelatin was added to all solutions (final concentration, 0.1%) to prevent the adsorption of ^{125}I-APP. Usually, 3–6 (or 6–12) μg of protein were incubated with ^{125}I-APP in a final volume of 200 μl (or 400 μl). Triplicate incubations were generally carried out at 25°C for 90–120 min and stopped by addition of 5 × 3 ml of cold buffer containing 0.1% bovine serum albumin, followed by rapid filtration through glass fiber filters (GF/B) treated with 0.3% polyethyleneimine. The trapped radioactivity was assayed with a LKB γ counter (82% efficiency). Addition of the ligand and filtration were carried out under dim light, and incubation was carried out in the dark. The free-ligand concentration was calculated as the difference between radioactivity added and membrane-bound radioactivity and was corrected for the loss due to adsorption onto the tubes (around 5%).

Photoaffinity Labeling. All experiments were performed in polyallomer tubes (Beckman). Membranes, diluted in 50 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing 0.1% gelatin, were incubated with 40–70 pM ^{125}I-APP in a total volume of 10 ml (15–30 μg of protein per ml) in the dark for 90–120 min at 25°C or 16 hr at 4°C. Nonspecific binding was determined in the presence of 0.2 μM mianserin. At the end of the incubation, a sample was used to perform an ^{125}I-APP binding assay in the dark. Membranes were centrifuged at 32,000 × *g* for 20 min, and pellets were washed with 10 ml of buffer containing 0.1 mM *p*-aminophenylacetic acid as a scavenger and recentrifuged. The pellets were resuspended in the supplemented buffer and were irradiated for 3 min in Petri dishes (10-cm diameter) maintained at a distance of 7 cm from a 366-nm UV light source (Ultraviolet Products, San Gabriel, CA; 150 watts). A sample of the irradiated membranes was retained for an ^{125}I-APP binding assay to determine membrane-bound radioactivity, and the remaining membranes were centrifuged at 32,000 × *g* for 20 min. The supernatant was discarded, and the pellets were either stored at −20°C or used immediately for electrophoresis. In experiments performed with protease inhibitors, the buffer was supplemented with the mixture of compounds described in *Membrane Preparation*.

$NaDodSO_4$/PAGE. Labeled membranes were treated with 200 μl of phosphate buffer containing 10% glycerol, 10% $NaDodSO_4$, 10 mM EDTA, and 5% 2-mercaptoethanol (except when stated), the mixture being maintained in a water bath at 37°C for 20 min. Bromophenol blue (0.001%) was added just before electrophoresis. When used, protease inhibitors were added in a concentrated solution to the solubilization buffer. Membranes (0.1 mg of protein) were electrophoresed on a 2-mm-thick polyacrylamide slab gel (11% or 5% when stated) at a current of 5 mA per lane (16). The gel was stained in 0.025% Coomassie blue R 250 dissolved in MeOH/AcOH, 45:9 (vol/vol), destained in MeOH/AcOH, 22:11, and dried. Autoradiography was performed (X.A.R. films, Eastman Kodak) at −80°C with intensifying screens, and the autoradiograms were scanned with a densitometer (Ultrascan XL, LKB). In some instances, the stained dried gel was cut into 2-mm slices, and radioactivity was determined with a γ counter.

RESULTS

Characterization of ^{125}I-APP Binding at Equilibrium. At 25°C and in the dark, binding of ^{125}I-APP (130 pM) to cerebellar membranes (10 μg of protein) achieved equilibrium after a 90-min period as evaluated by the filtration assay (data not shown). Specific binding at equilibrium, evaluated as the difference between total and nonspecific binding measured in the presence of 0.2 μM mianserin, an H_1-receptor antagonist, was saturable. Nonspecific binding varied linearly with the ^{125}I-substituted ligand concentration and represented about 30% of the total at concentrations <30 pM (Fig. 2). Computer analysis of the specific binding using a one-site model (17) gave a K_d of 1.2 ± 0.4 × 10^{-11} M, a B_{max} of 237 ± 16 fmol/mg of protein, and a Hill

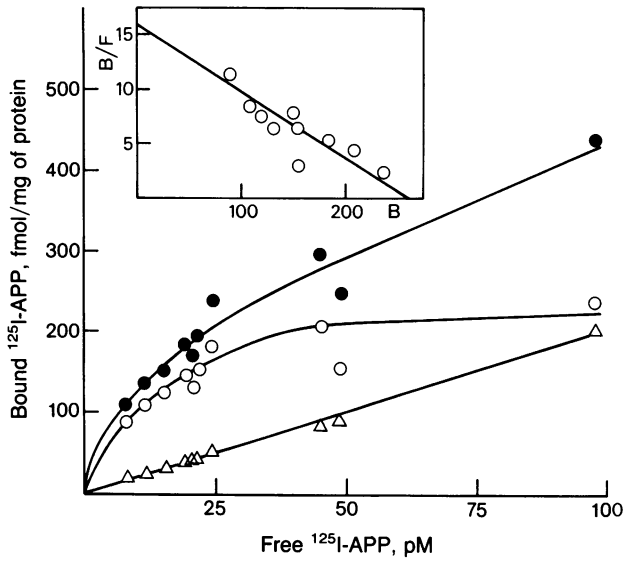

FIG. 2. Saturation of ^{125}I-APP binding to guinea pig cerebellar membranes. Membranes (3 μg of protein per 0.2 ml) were incubated for 90–120 min at 25°C in the dark with ^{125}I-APP. Nonspecific binding was measured in the presence of 0.2 μM mianserin. Data are means of triplicate determinations in a single typical experiment, representative of three such experiments. ●, Total binding; ○, specific binding; △, nonspecific binding. (*Inset*) Scatchard plot for saturation of specific binding. B, bound; F, free.

coefficient of 1.0 ± 0.3 (means ± SEM of values derived from three separate experiments). Scatchard analysis of the same data afforded similar values (Fig. 2 *Inset*).

Specific binding of ^{125}I-APP was inhibited in a concentration-dependent manner by various H_1-receptor antagonists, whereas H_2-receptor antagonists were poorly effective. K_i values of competitors were calculated from their IC_{50} and the K_d of ^{125}I-APP (Table 1).

Photoaffinity Labeling with ^{125}I-APP and NaDodSO$_4$/PAGE Analysis. After UV-irradiation of cerebellar membranes previously equilibrated with 50 pM ^{125}I-APP, filtration assays indicated that about 50% of the initially bound radioactivity remained attached to membranes (not shown). Subsequent solubilization and NaDodSO$_4$/PAGE analysis of these membranes on an 11% polyacrylamide gel indicated that a fraction of this radioactivity was covalently incorporated.

Three main bands were consistently labeled in standard experiments performed in the absence of protease inhibitors, 2-mercaptoethanol being present from the solubilization step. Covalent labeling of one of these bands (92 ± 2 kDa), which represented about 5% of the gel radioactivity, was only partially prevented by 0.2 μM mianserin. In contrast, labeling of the other two main bands [55.6 ± 0.5 and 47.3 ± 0.4 kDa (means ± SEM of eight different determinations)] was completely prevented by 10 nM mianserin, half-protection occurring at about 0.1 nM (not shown). When the stained, dried gels were sliced at the level of these two bands, their radioactivity was found to represent only 4–6% of the radioactivity bound to irradiated membranes. When higher concentrations of membranes (70 instead of 15–30 μg of protein per ml) and ligand (200 instead of 50 pM) were used, more bands were covalently labeled, but only the bands at 47 and 56 kDa were protected by 0.2 μM mianserin. In particular, the 92-kDa band, which under these conditions was approximately as heavily labeled as the 47-kDa and 56-kDa bands, was not protected by up to 10 μM mianserin (not shown).

Effects of Protease Inhibitors and 2-Mercaptoethanol on the Photoaffinity-Labeling Pattern. When protease inhibitors were present during the whole experiment from membrane preparation to NaDodSO$_4$/PAGE, the relative amounts of label in the two bands (56 and 47 kDa) was modified (Fig. 3, lanes 1–4), the ratio of their radioactivities becoming 65:35 instead of 40:60 in their absence (values obtained from autoradiogram scannings). In contrast, the relative labeling of the 92-kDa band was apparently not modified in the presence of protease inhibitors. The relative importance of the 47-kDa band was also increased at the expense of the 56-kDa band when the duration of the binding incubation in the absence of protease inhibitors was increased to 18 hr at 25°C or 4°C (not shown).

Omission of 2-mercaptoethanol from the solubilization mixture led to the appearance of an additional band of higher molecular mass (>200 kDa), apparently at the expense of the two light bands (56 and 47 kDa) which were, however, still present (Fig. 3, lanes 5–8). This heavy band was seen in the presence or absence of protease inhibitors, and its labeling was completely prevented in the presence of 0.2 μM mianserin.

Several H_1-receptor antagonists used at low concentrations (corresponding to 2–5 times their K_is), partially prevented the labeling of the 47-kDa and 56-kDa peptides

Table 1. Characterization of ^{125}I-APP reversible binding to guinea pig cerebellar membranes: Inhibition by histamine receptor antagonists as compared with inhibition of [^{125}I]iodobolpyramine binding and of histamine-induced contraction of the guinea pig ileum *in vitro*

		Cerebellar binding	
	Guinea-pig ileum contraction,* K_B (nM)	[^{125}I]Iodobolpyramine,† K_i (nM)	^{125}I-APP, K_i (nM)
Mepyramine	0.4	0.33	0.13
Triprolidine	0.1	0.22	0.43
Mianserin	2.0	0.22	0.16
(+)-Chlorpheniramine	0.5	0.21	0.21
(−)-Chlorpheniramine	14	35	59
Cimetidine	446,000	>27,000	>32,000
Ranitidine			>32,000
Burimamide	228,000	112,000	>32,000

^{125}I-APP (25 pM) was incubated with cerebellar membranes, and the various drugs and binding assays were performed in the dark. IC_{50} was converted into K_i by using the equation $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of ^{125}I-APP and K_d is its equilibrium dissociation constant (12 pM). Each drug was tested in at least two separate experiments at four to eight different concentrations. K_B, apparent dissociation constant.

*Ref. 18.

†Ref. 4.

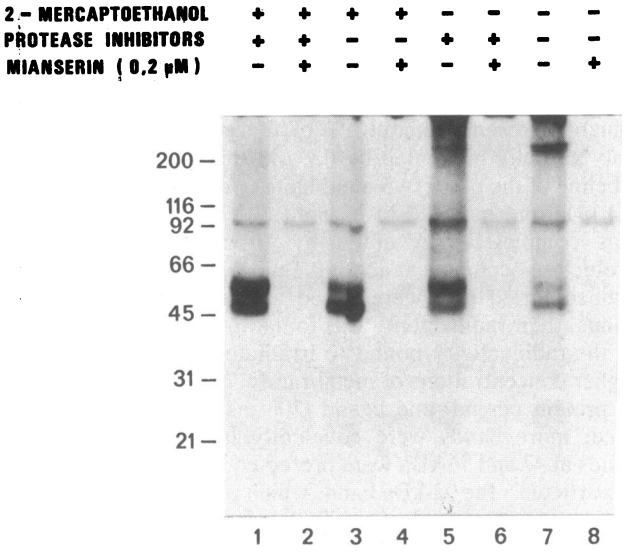

FIG. 3. Effects of protease inhibitors and 2-mercaptoethanol on the photoaffinity labeling pattern. Membranes were incubated for 90–120 min at 25°C with the ligand (50–70 pM) in buffer alone (lanes 1, 3, 5, and 7) or together with 0.2 μM mianserin (lanes 2, 4, 6, and 8). Protease inhibitors were present (+) or absent (−) throughout the various steps of the experiment. Solubilization was performed in the presence (+) or absence (−) of 5% 2-mercaptoethanol. The whole experiment was performed on the same day, and specific binding did not significantly differ under the various conditions tested. Molecular mass is shown × 10^{-3}.

whereas, at high concentrations, labeling was totally prevented (Fig. 4).

The photoaffinity-labeling pattern of cerebellar membranes solubilized in the absence of 2-mercaptoethanol was also analyzed by NaDodSO$_4$/PAGE on a 5% polyacrylamide gel instead of an 11% gel to provide a more precise estimate of the apparent molecular mass of the heaviest band. Autoradiography of the gel again revealed the four main bands: one (94 ± 5 kDa) whose labeling was partially inhibited by mianserin and the three others (49 ± 3 kDa, 59 ± 3 kDa, and 350–400 kDa) whose labeling was completely inhibited by mianserin (Fig. 5). Again the heaviest band was no longer apparent when solubilization was performed in the presence of 2-mercaptoethanol.

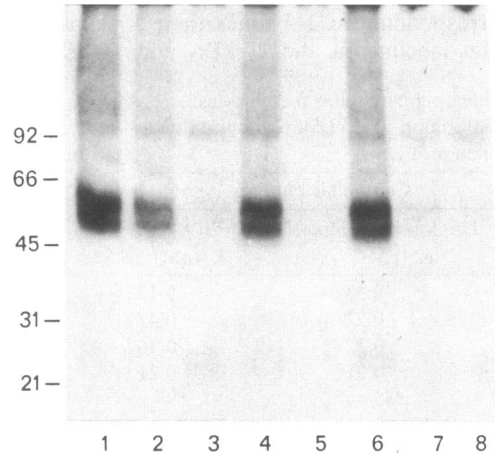

FIG. 4. Effects of various H_1-receptor antagonists on the photoaffinity labeling pattern studied with protease inhibitors being present throughout the experiment. Membranes were incubated for 90–120 min at 25°C with 40–60 pM ^{125}I-APP alone (lane 1) or in the presence of 1 nM or 50 nM triprolidine (lanes 2 and 3), 1 nM or 50 nM (+)chlorpheniramine (lanes 4 and 5), 50 nM or 2.5 μM (−)-chlorpheniramine (lanes 6 and 7), or 0.2 μM mianserin (lane 8). Molecular mass is shown × 10^{-3}.

DISCUSSION

The present study demonstrates the effectiveness of ^{125}I-APP for photoaffinity labeling H_1-receptor peptides from guinea pig cerebral membranes. Like the reversible probe iodobolpyramine (4), this compound belongs to a series modeled on the potent H_1-receptor antagonist mepyramine. In this series of compounds (5), an amino group, optimally separated by a chain of five carbon atoms from the tertiary amino group of mepyramine, serves to introduce various functional groups via amidification such as, in the present case, a *p*-aminophenylacetyl residue. Then an ^{125}I atom can be easily introduced into this part of the molecule, and the aromatic amino group, converted into an azido group.

This approach was successful in producing a compound of high specific radioactivity with high affinity and selectivity for the H_1 receptor. In the absence of light, ^{125}I-APP binding apparently occurred to a single population of sites with a K_d at equilibrium of 1.2 ± 0.4 × 10^{-11} M, suggesting that this compound is among the most potent H_1-receptor antagonists known so far. That these sites correspond to H_1 receptors is indicated by their density in cerebellar membranes (B_{max} = 237 ± 16 pmol/mg of protein) similar to that of [^{3}H]mepyramine (7) or [^{125}I]iodobolpyramine binding sites (4). In addition, ^{125}I-APP binding was inhibited by various H_1-receptor antagonists in a stereoselective fashion and with K_i values in good agreement with corresponding values for inhibition of [^{3}H]mepyramine or [^{125}I]iodobolpyramine binding (4) and with the published apparent dissociation constants for inhibition of histamine-induced contractions of the guinea pig ileum, a reference H_1 receptor-mediated response (18) (Table 1). It is noteworthy that the nonspecific binding of ^{125}I-APP was quite low, representing <30% of the total at concentrations below twice the K_d.

After UV-irradiation, a fraction of the bound radioactivity remained covalently attached to H_1-receptor subunits. The extent of photoincorporation calculated from the amount of specifically bound ligand prior to photolysis versus that estimated from NaDodSO$_4$ gel slices, was about 5%. Among the various bands revealed by autoradiography of NaDodSO$_4$/PAGE gels, two peptides with apparent molecular masses of 47–49 kDa and 55–59 kDa appeared consistently

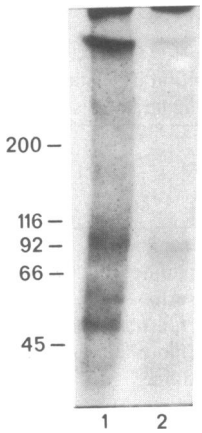

FIG. 5. Photoaffinity labeling pattern established in the absence of 2-mercaptoethanol and protease inhibitors using 5% polyacrylamide gel electrophoresis. Membranes were incubated for 90–120 min at 25°C in the presence of 50 pM ^{125}I-APP in the absence (lane 1) or the presence (lane 2) of 0.2 μM mianserin. The experiment was repeated twice with similar results. Molecular mass is shown × 10^{-3}.

and were specifically labeled under various experimental conditions (i.e., in the presence or absence of 2-mercaptoethanol and/or protease inhibitors). These two peptides were labeled in membranes of cerebellum as well as of other brain regions in which H_1 receptors are present (not shown). Photoincorporation was completely prevented by 0.2 μM mianserin, an H_1-receptor antagonist chemically unrelated to the ^{125}I-substituted ligand, and half-protection occurred at about 0.1 nM mianserin. Photoincorporation was also prevented by triprolidine and, in a stereoselective manner, by the two chlorpheniramine enantiomers, indicating that the labeling occurred with an H_1-receptor specificity. Interestingly, in the presence of protease inhibitors, labeling of the 55- to 59-kDa peptide was increased at the expense of the lower peptide, strongly suggesting that the latter was generated by hydrolysis of the former under the action of endogenous proteases of the membrane preparations. We propose that the 55- to 59-kDa peptide represents the binding subunit of the histamine H_1 receptor.

A 90- to 94-kDa peptide also was photolabeled under the various conditions (particularly at high ligand concentrations) but, in contrast with the two lighter ones, its labeling was prevented only in a partial and inconsistent manner by mianserin, and protection required concentrations of the latter above 10 nM (i.e., notably higher than the K_i). These observations cast some doubt on the idea that this peptide might represent a binding subunit of the H_1 receptor.

Finally in nonreducing conditions (i.e., in the absence of 2-mercaptoethanol), a major band that minimally penetrated standard NaDodSO$_4$/PAGE gels (molecular mass > 200 kDa) appeared, apparently at the expense of the two 47- to 49-kDa and 55- to 59-kDa peptides. Labeling of this band was completely prevented in the presence of mianserin. When more porous gels (5% polyacrylamide instead of 11%) were used, also in the absence of 2-mercaptoethanol, the migration of this band was compatible with a molecular mass of 350–400 kDa. This value must be regarded as an estimate, however, because many possible errors attend the determination of molecular masses of large glycoprotein complexes with NaDodSO$_4$/PAGE gels.

It is tempting to speculate that this band represents the native H_1 receptor in which the 56- to 59-kDa peptide could correspond to the histamine-binding peptide linked to one or more other peptides by one or more disulfide bonds. However, it cannot be excluded that the appearance of the 350- to 400-kDa band results from artifactual formation of disulfide links during the preparation and solubilization of membranes in nonreducing conditions. Furthermore, the molecular mass of 350–400 kDa does not agree with molecular mass values of the H_1 receptor previously obtained by other approaches, although this point cannot be considered to be settled. Sucrose gradient-centrifugation and gel-filtration experiments have indicated a mass of 430 kDa for the receptor–digitonin complex obtained by solubilization of guinea pig cerebral membranes, but the molecular mass of the receptor protein itself could not be deduced (11). Target size analysis of the [^{3}H]mepyramine binding sites led to a molecular mass of 160 kDa in bovine and human cerebral cortex (9) and of 107 kDa in guinea-pig liver (10). Obviously these two values are notably different from the mass of the large complex evidenced by NaDodSO$_4$/PAGE, but the identity of these sites as H_1 receptors remains to be more firmly established, particularly in liver, a tissue in which the inhibitory potency of antagonists was significantly lower than at reference biological systems.

The present studies strongly suggest that at least part of the ligand binding domain of the H_1 receptor resides within a 55- to 59-kDa peptide, but further studies are required to clarify its relationship to the whole receptor.

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